Short Communication

Olive pomace oil can be used as an alternative carbon source for clavulanic acid production by *Streptomyces clavuligerus*

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Abstract

Clavulanic acid is an important drug, both medically and economically. It is used to combat bacterial resistance to β-lactam antibiotics and is on the World Health Organisation's List of Essential Medicines in combination with amoxicillin. An olive oil industry waste product, olive pomace oil (OPO), is a potential alternative carbon source for clavulanic acid production by *Streptomyces clavuligerus*. Olive pomace oil is six times cheaper than glycerol, which is the current industry standard. The aims of this study were to examine if olive pomace oil can be used as a carbon source for clavulanic acid production and to compare the clavulanic acid yield achieved in shake flasks and 1.8 L bioreactors. It was observed that olive pomace oil was efficiently utilised as a sole carbon source by *S. clavuligerus* growing in a P-limited medium. The *S. clavuligerus* cells grew faster in olive pomace oil-containing cultures compared to the glycerol-containing cultures (control) and produced comparable levels of clavulanic acid, but much earlier. In cultures with ISP2 medium that contained glycerol or olive pomace oil, higher levels of

clavulanic acid were obtained in shake flask cultures with olive pomace oil. Interestingly, the same levels of clavulanic acid were observed in oil-containing cultures in bioreactors, but 48 h earlier. Furthermore, the oil-containing cultures did not need addition of an antifoam agent, while higher levels of cell viability were maintained after 72 h in these fermentations compared to the cultures that contained glycerol. Our results suggest that olive pomace oil can replace glycerol for clavulanic acid production in *S. clavuligerus* fermentations, which will significantly increase the productivity and cut the cost for industrial clavulanic acid biosynthesis. The same carbon source can be tested in other similar fermentation approaches for the production of antibiotics or other valuable bioproducts.

Keywords: Antibiotics; Bioprocessing, Clavulanic acid; Olive pomace oil; *Streptomyces*; Waste.

Statement of Novelty

Olive pomace oil is an environmentally toxic waste of the olive oil industry. We show that it can replace glycerol in microbial fermentations, leading to higher yield of clavulanic acid production.

Introduction

The most common mechanism by which bacteria acquire resistance to beta-lactam antibiotics is through the production of beta-lactamase enzymes. These enzymes cleave the beta-lactam rings of important antibiotics such as penicillins and cephalosporins, leading to their inactivation. Clavulanic acid, produced by *Streptomyces clavuligerus*, is used in conjunction with β-lactam antibiotics, acting as a suicide inhibitor that protects these drugs from the β-lactamases of resistant bacteria [1]. The combination of amoxicillin and clavulanic acid is on the World Health Organisation (WHO) list of essential medicines [2]. Due to the importance of this treatment, much research has been done to improve the growth of *S. clavuligerus*, and the organism's production of clavulanic acid [3].

Different from most organisms, *S. clavuligerus* lacks a glucose transport system [4], so it is unable to utilise this sugar. Instead, glycerol is the most popular carbon source used for industrial production of clavulanic acid [5]. Glycerol is, however, relatively expensive, so cheaper alternatives such as oils have been looked into. It has been shown previously that olive oil outperforms glycerol (and other oils) in terms of the concentration of clavulanic

acid being produced by *S. clavuligerus*, with olive oil producing a maximum concentration of 47 mg L^{-1}, almost double that of the standard glycerol (25 mg L^{-1}) [6].

Olive mills generate huge waste in short periods of time, which is seen as an environmental problem for Mediterranean countries [7]. These wastes have high phytotoxicity, with studies also showing adverse effects on soil microbial populations [8] and aquatic ecosystems [9]. While olive oil is cheaper than glycerol, and has been shown to work better, it does not seem ethical to add to existing demand, and in turn, increasing waste. One of these wastes from the olive oil industry, olive pomace oil (OPO) [10], has its own potential as a carbon source for *S. clavuligerus*. Miranda *et al*. [11] show that there is around 52% carbon composition in the pomace, while the pulp (where regular olive oil is obtained) has around 55%. This suggests that OPO likely has sufficient energy to be a viable carbon source, still outperforming glycerol as olive oil was shown to. The average price of OPO is £1.50 per L, which is six times less expensive than the standard carbon source glycerol (£9 per L). In addition to being cost effective, OPO utilisation would also create a positive reuse for some of the vast amounts of waste found in the olive oil industry. If accepted widely, this could bring huge economic and environmental benefits to Mediterranean countries, and lower costs for clavulanic acid producers. Additionally, oil has natural anti-foaming properties and can enhance secondary metabolism [12].

Lots of efforts have been devoted to the development of *S. clavuligerus* strains with the aim to improve the production of clavulanic acid. These strain improvements, whether they were done through random mutagenesis [13] or targeted modification of metabolic pathways [14], were usually carried out with glycerol in mind as the carbon source. With food oils already yielding higher clavulanic acid production, and in some cases culture growth, strain improvement of *S. clavuligerus* to increase the utilisation of these oils [15] could greatly improve clavulanic acid production.

Although complex media are commonly used for industrial clavulanic acid production, defined media allow us to test varying media ingredients. In this paper, we used defined media to test the growth of *S. clavuligerus* and the yield of clavulanic acid using OPO as the sole carbon source. To assess the fermentation efficiency of different carbon sources, glycerol was set as control to produce clavulanic acid. Because of the high carbon content, we hypothesise that OPO will prove to be a suitable carbon source, likely more effective than the industry standard of glycerol for clavulanic acid production. In addition, we believe that OPO will continue to be effective at higher volumes as part of complex media.

Materials and Methods

Strains and media

The bacterial strain used in this study was *Streptomyces clavuligerus* DM738 (kindly provided by Prof. Paul Hoskisson, University of Strathclyde, UK). The medium used for the pre-culture was SV2, containing (per L): Glycerol, 15 g; Glucose, 15 g; Peptone, 15 g; $CaCO_3$, 1 g. For the flask cultures, a P-limited medium was used, containing (per L): NH_4Cl, 7 g; KH_2PO_4, 0.25 g; MOPS, 21 g. The pH value was adjusted to 6.8 and the medium was autoclaved at 121°C for 15 min. 10 mL of trace elements stock solution was aseptically added per L of P-limited medium after autoclaving [6]. The trace elements stock solution contained (per L): $MgSO_4.7H_20$, 25 g; $FeSO_4.7H_2O$, 2.5 g; $CoCl_2$, 0.055 g; $CuCl_2$ 0.053 g, $CaCl_2.2H_2O$, 1.4 g; $ZnCl_2$, 1 g; $MnCl_2$, 1 g; Na_2MoO_4, 0.03 g. For shake flask and bioreactor cultures, ISP2 medium was used, containing (per L): Yeast extract, 4 g; Malt extract, 10 g; Dextrose, 4 g. The carbon source was added at 0.6% v/v by filter sterilisation. All media were autoclaved at 121°C for 15 min unless stated otherwise.

Inoculum preparation and cultures

For the pre-culture, 1 mL of spores was added into 50 mL of SV2 in a 250 mL glass baffled flask and agitated at 200 rpm at 30°C for 48 h. The culture was transferred into plastic universals and centrifuged at 6000 xg for 5 min. The supernatant was removed, and the pellets were resuspended in 6 mL of P-limited media. For the shake flask experiments, 2 L glass baffled flasks were used containing 400 mL of medium (P-limited or ISP2). Each flask was inoculated with 1 mL of the re-suspended inoculum. The flasks were incubated at 30°C, with agitation at 150 rpm.

For the bioreactor cultures, 1.8 L bioreactors (DASGIP parallel system, Eppendorf, UK) were used. Each vessel contained 1 L of ISP2 medium. The inoculum was prepared as described above and introduced aseptically with a syringe (5% v/v). The agitation rate was set at 1000 rpm, with a culturing temperature of 30°C. The pH value was maintained at 7 and the aeration rate was set at 0.25 L min^{-1}. OPO or glycerol were added at the same percentage as above. Filter-sterilised Antifoam 204 (Sigma-Aldrich, UK) was added at 0.001% v/v in the glycerol-containing cultures.

Clavulanic acid assay

Clavulanic acid levels were determined as described by Bird *et al*. [16].

Biomass determination

Biomass levels were determined as described by Efthimiou *et al*. [6].

Results and Discussion

Production of many secondary metabolites by *Streptomyces* species, including clavulanic acid, are negatively regulated by phosphate [17]. P-limited media is thus often used to stimulate the production of these compounds. As can be seen in Figure 1A, in P-limited medium, *S. clavuligerus* reached maximal growth five days earlier than in the oil-containing cultures compared to the glycerol containing cultures. The OPO cultures also produced higher levels of clavulanic acid until 264 h with a maximum clavulanic acid concentration of nearly 150 mg L^{-1}. The maximum clavulanic acid production in the glycerol cultures was over 200 mg L^{-1}, however this decreased later. These results are a good first indication that OPO could be a suitable carbon source replacement for clavulanic acid production in *S. clavuligerus*. It should be noted that the method of inoculation ensures a minimal carry over of nutrients.

Once it was determined that OPO was able to be utilised in a defined medium, it was then added as a carbon source in a complex media (Figures 2A and 2B), which more accurately replicates the conditions in industrial productions (albeit at a laboratory scale). In the first 24 h of growth in complex media, OD values obtained were very similar between the carbon sources, but OPO cultures achieved higher growth at all time points recorded. In addition, Figure 2B shows that OPO as a carbon source in complex media again led to the production of much larger amounts of clavulanic acid (over double that of glycerol at 48 h). This is similar to previous results obtained comparing clavulanic acid production in olive oil and glycerol [6, 18]. Cell viability was also greater in OPO cultures across all experiments when spread onto plates (data not shown).

The next step was to scale up the experiment to 1.8 L bioreactors, further replicating industrial production. Figure 2C continues to support OPO as a superior carbon source to glycerol, with the OPO cultures obtaining a higher maximum biomass, earlier than the glycerol culture. In Figure 2D, it can be seen that clavulanic acid production

also occurs earlier in OPO cultures with a maximum of 325 mg L^{-1} at 48 h. This is achieved 48 hours earlier than the maximum 350 mg L^{-1} found in glycerol cultures.

Using data from the bioreactor cultures, Table 1 shows that OPO outcompetes glycerol in all four aspects measured: growth yield, growth rate, clavulanic acid yield, clavulanic acid rate. Growth yield and growth rate values from OPO cultures were almost double that of glycerol cultures. Clavulanic acid yield between carbon sources was closer, but OPO still appears the more favourable than glycerol (59.96 mg g^{-1} of carbon source and 46.16 mg g^{-1} of carbon source, respectively). Similarly, the rate of clavulanic acid production was 1.47 times higher in OPO cultures than in glycerol cultures.

The effectiveness of OPO also has the potential to drastically increase through strain improvement targeted specifically at the use of this carbon source. Industrial strains reportedly have a clavulanic acid production nearing 3 g L^{-1} which is far higher than wild type strains (around 25-120 mg L^{-1}) [3]. Spontaneous mutations with oil as a carbon source have been recorded [19], proving that this is viable. In combination with targeted mutagenesis, there is an even greater potential for OPO. It would be interesting to see the levels of clavulanic acid produced by these industrial strains when OPO is the carbon source.

It was also observed in these experiments that OPO acted as a strong antifoam, leading to negligible foaming of the media compared to glycerol. This might partly explain the better results of OPO in the shake flask experiments (antifoam was added to glycerol cultures in the bioreactor experiments). Foam in the glycerol cultures may have impeded on gas exchange, negatively impacting on growth and clavulanic acid production [20]. Foam is generally seen as undesirable in the production of antibiotics [21].

All three of these experiments indicate that OPO is a superior carbon source as it led to faster growth, faster clavulanic acid production, and removed the need for an antifoam – an additional cost when glycerol is used. In other studies, fed-batch cultures have been shown to further increase the levels of clavulanic acid production [22].

This by-product of the olive oil industry is extracted from undesirable waste such as the skin, pulp, stone, and kernel of olives [23]. By providing these waste products with a use, OPO extraction both alleviates environmental pollution and can provide a further source of revenue for olive oil-producing countries. This has the potential to boost the economies of Spain, Greece, Italy, and other Mediterranean countries. OPO as a carbon source could also be of great economic impact for clavulanic acid producers as a result of its low cost of around £1.50 per L litre, six times cheaper than glycerol.

There are disadvantages that should be taken into account. First, there is often oil residue at the end of the culture due to limited interaction of the bacteria and the lipid droplets [12]. However, this problem is often countered by slowly deeding the oil during culture [24]. Secondly, there is often a greater oxygen demand in cultures with oils compared to carbohydrates [12].

In conclusion, our study shows that OPO is an effective carbon source for clavulanic acid production by *S. clavuligerus* fermentation. This extremely cheap by-product of the olive oil industry outperformed the standard carbon source, glycerol, by promoting faster growth, larger quantities of clavulanic acid production, and natural antifoaming properties. We believe that by switching to OPO, industrial producers could save large amounts of money, while at the same time achieving higher clavulanic acid yield. This would not only hugely benefit the Mediterranean countries that produce OPO, but reduce production costs of clavulanic acid, bringing the price down of an essential drug. Finally, it is possible that the same carbon source can be used in other similar fermentation approaches for the production of antibiotics or other valuable bioproducts.

Conflicts of Interest

The authors declare no conflict of interest.

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Table Legend

Table 1 Biomass and clavulanic acid production yields and rates (curve slope values), with olive pomace oil and glycerol as carbon sources (calculated using data from the bioreactor cultures).

Figure Legends

Figure 1 Growth (A) and clavulanic acid production (B) in shake flasks with P-limited medium and glycerol (gray line) or olive pomace oil (black line) as sole carbon source (n = 3, where n is the number of biological replicates). CLA: clavulanic acid. Error bars: ± Standard Deviation.

Figure 2 Growth (A) and clavulanic acid production (B) in shake flasks with ISP2 and glycerol (gray line) or olive pomace oil (black line) as additional carbon source. Growth (C) and clavulanic acid production (D) in 1.8 L DASGIP bioreactors with ISP2 and glycerol or olive pomace oil as additional carbon source (n = 3, where n is the number of biological replicates). CLA: clavulanic acid. Error bars: ± Standard Deviation.

Figure 1

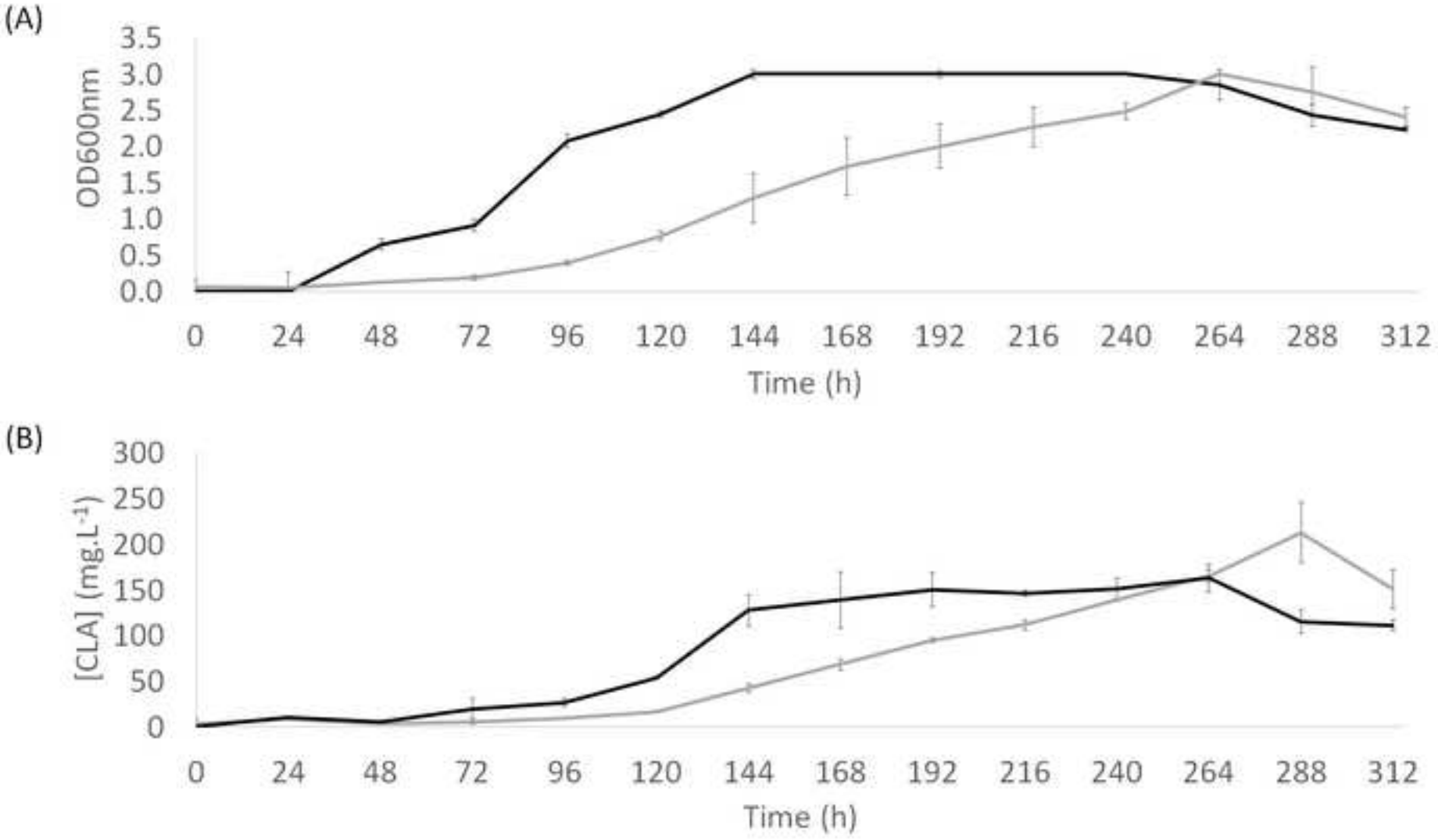

Figure 2

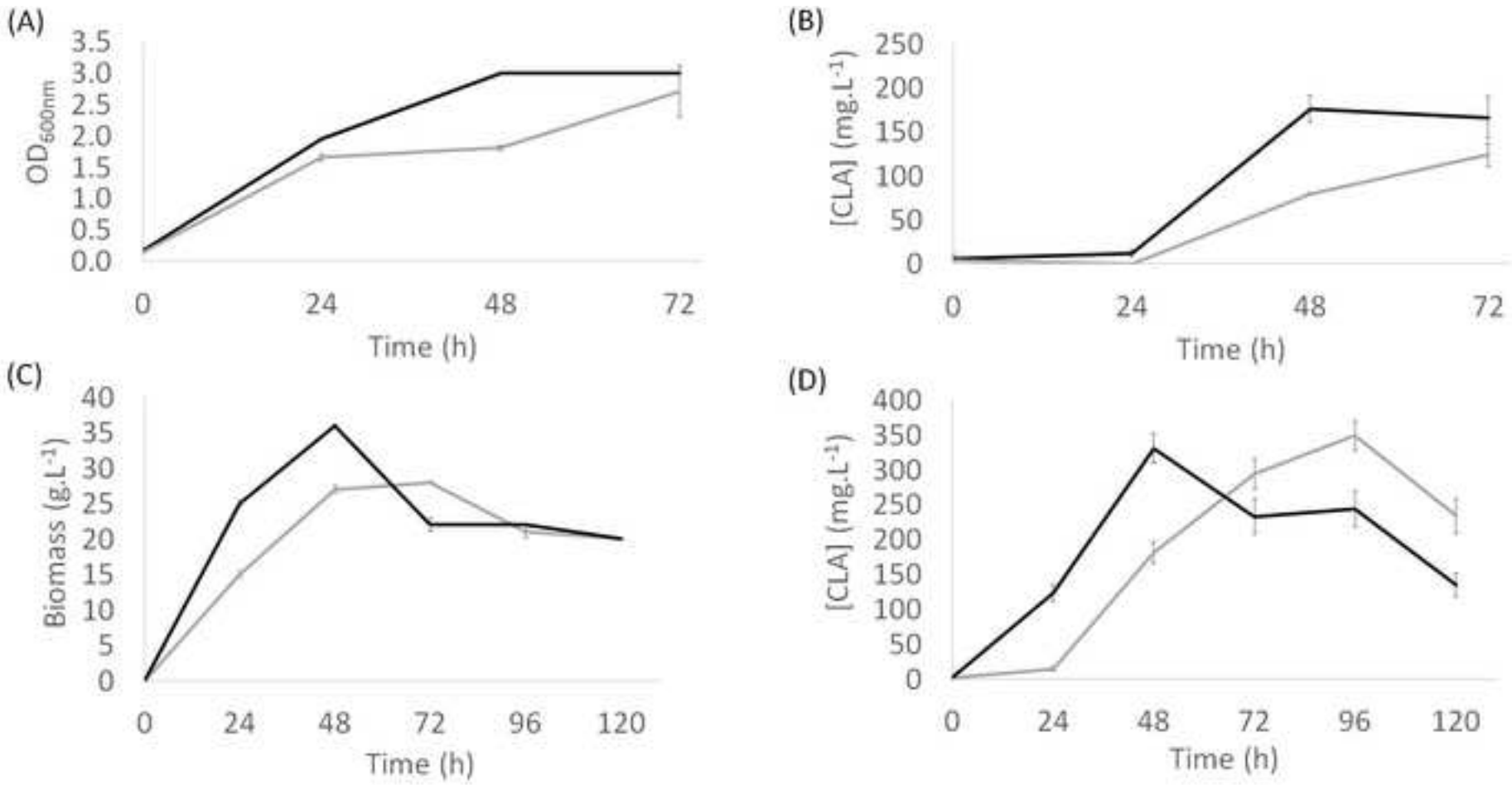

Table 1. Biomass and clavulanic acid production yields of carbon sources

Carbon source	Growth yield (g g^{-1} of carbon source)	Growth rate ($g \cdot L^{-1} \cdot h^{-1}$)	Clavulanic acid yield (mg g^{-1} of carbon source)	Clavulanic acid rate ($mg \cdot L^{-1} \cdot h^{-1}$)
Olive pomace oil	6.52	0.75	59.96	6.87
Glycerol	3.70	0.40	46.16	4.66

Graphic abstract

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